Heligmosomoides polygyrus Promotes Regulatory T-Cell Cytokine Production in the Murine Normal Distal Intestine[▽]

Tommy Setiawan,¹ Ahmed Metwali,² Arthur M. Blum,¹ M. Nedim Ince,² Joseph F. Urban, Jr.,³ David E. Elliott,^{2*} and Joel V. Weinstock¹

Division of Gastroenterology-Hepatology, Tufts-New England Medical Center, 750 Washington Street, Boston, Massachusetts 02111¹; Division of Gastroenterology-Hepatology, Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242-1009²; and Nutrient Requirements & Functions Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Bldg. 307C, Rm. 213, BARC-East, Beltsville, Maryland 20705³

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Helminths down-regulate inflammation and may prevent development of several autoimmune illnesses, such as inflammatory bowel disease. We determined if exposure to the duodenal helminth *Heligmosomoides polygyrus* establishes cytokine pathways in the distal intestine that may protect from intestinal inflammation. Mice received 200 *H. polygyrus* larvae and were studied 2 weeks later. Lamina propria mononuclear cells (LPMC) were isolated from the terminal ileum for analysis and in vitro experiments. Mice with *H. polygyrus* were resistant to trinitrobenzenesulfonic acid (TNBS)-induced colitis, a Th1 cytokine-dependent inflammation. *Heligmosomoides polygyrus* did not change the normal microscopic appearance of the terminal ileum and colon and minimally affected LPMC composition. However, colonization altered LPMC cytokine profiles, blocking gamma interferon (IFN- γ) and interleukin 12 (IL-12) p40 release but promoting IL-4, IL-5, IL-13, and IL-10 secretion. IL-10 blockade in vitro with anti-IL-10 receptor (IL-10R) monoclonal antibody restored LPMC IFN- γ and IL-12 p40 secretion. IL-10 blockade in vivo worsened TNBS colitis in *H. polygyrus*-colonized mice. Lamina propria CD4⁺ T cells isolated from colonized mice inhibited IFN- γ production by splenic T cells from worm-free mice. This inhibition did not require cell contact and was dependent on IL-10. *Heligmosomoides polygyrus* colonization inhibits Th1 and promotes Th2 and regulatory cytokine production in distant intestinal regions without changing histology or LPMC composition. IL-10 is particularly important for limiting the Th1 response. The T-cell origin of these cytokines demonstrates mucosal regulatory T-cell induction.

Epidemiological evidence suggests that environmental factors contribute strongly to the risk of acquiring inflammatory bowel disease (IBD). In developed countries during the 1940s to 1980s, the incidence of Crohn's disease (CD) rose dramatically within a single generation (9). CD is rare in less-developed countries but emerges as these countries become industrialized (19). Now CD is common in developed countries with temperate climates, where the rates of colonization with helminthic parasites rapidly declined after the 1940s (12). Helminths alter their host's immune responses (6), which may help limit development of potentially pathological-type inflammations like IBD (12, 32).

Mice rectally exposed to trinitrobenzenesulfonic acid (TNBS) develop colitis that is prevented by inhibiting Th1 cytokines (interleukin 12 [IL-12], gamma interferon [IFN- γ], and tumor necrosis factor alpha [TNF- α]) (22). IL-10 and transforming growth factor beta (TGF- β) naturally down-regulate the inflammation (15). Previously we showed that mice systemically exposed to nonviable eggs of the helminthic parasite *Schistosoma mansoni* are resistant to TNBS-induced colitis (13). This resistance to TNBS colitis requires IL-4 and

intact Stat 6 circuitry and is associated with depressed IFN- γ and augmented IL-10 colonic mRNA levels.

Patients with IBD show clinical improvement after exposure to the whipworm *Trichuris suis* (27–29). This suggests that colonization with intestinal helminths modifies mucosal immune responses and inhibits inflammation. We studied *Heligmosomoides polygyrus*, a helminth that naturally colonizes mice, to delineate the potential mechanisms of human disease modification. *Heligmosomoides polygyrus*, like *T. suis*, is strictly an intestinal organism that displays no systemic migration during its development in the host. Unlike *T. suis*, *H. polygyrus* inhabits the duodenal mucosa. Mice ingest larvae that briefly inhabit the duodenal wall and then reenter the lumen to mature into adult worms. Mice colonized with *H. polygyrus* have a blunted delayed-type hypersensitivity response to ovalbumin (25). Colonization with *H. polygyrus* induces lamina propria (LP) macrophages that have an alternatively activated phenotype (3, 24).

The aim of this investigation was to determine if exposure to the duodenal helminth *H. polygyrus* establishes cytokine pathways in distal intestinal mucosa of wild-type (WT) mice that could prove protective against intestinal inflammation. It was found that colonization of the duodenum with *H. polygyrus* inhibited Th1 and promoted Th2 and regulatory cytokine production by LP mononuclear cells (LPMC) from the distant intestine without altering mucosal histology or LPMC composition. Mucosal T cells were an important source of these

* Corresponding author. Mailing address: Division of Gastroenterology (4611 JCP), University of Iowa Hospital and Clinics, 200 Hawkins Drive, Iowa City, IA 52242-1009. Phone: (319) 353-8574. Fax: (319) 353-6399. E-mail: david-elliott@uiowa.edu.

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cytokines, suggesting induction of regulatory T cells in the intestine.

MATERIALS AND METHODS

Mice and *H. polygyrus* infection. This study used WT C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). Mice about at week 5 of age were colonized with 200 *H. polygyrus* third-stage larvae by oral gavage. Infective, ensheathed *H. polygyrus* third-stage larvae (U.S. National Helminthological Collection no. 81930) were obtained (from fecal cultures of eggs) by the modified Baermann method and stored at 4°C until used. Animals were housed and handled appropriately following national guidelines and as approved by our Animal Review Committee.

Induction of TNBS colitis. Colitis was induced by rectal instillation of TNBS (23). Briefly, control or parasite-exposed C57BL/6 mice were fasted for 20 h and then anesthetized. A 1.2-mm-diameter catheter was advanced 4 cm into the colon, and 0.10 to 0.12 ml of TNBS solution (5 mg/ml TNBS [Sigma] in 50% ethanol) was instilled. Animals then were held by the tail for 3 min to assure that the TNBS uniformly contacted the colonic mucosa. For some experiments, mice were given 0.5 mg of either rat immunoglobulin G (IgG) (Sigma) or monoclonal anti-IL-10R antibody (1B1.3) by intraperitoneal injection upon recovery from anesthesia. Animals were euthanized day 4 after TNBS challenge. Colitis was maximal at about day 4, and this time point was used in experiments analyzing the effect of helminthic exposure on intestinal inflammation. Investigators were blinded to the animal treatment group at the time of TNBS instillation to remove procedural bias.

Evaluation of mucosal inflammation. To grade intestinal inflammation, colons were removed at the indicated time point, rolled, fixed, and embedded in paraffin. The sections were stained with hematoxylin and eosin. Two pathologists in a blinded fashion graded the degree of colonic inflammation from 0 to 4. The scoring system was as follows: 0, no inflammation; 1, low-level inflammation; 2, intermediate level; 3, high-level inflammation with wall thickening; 4, transmural infiltration, loss of goblet cells, and wall thickening (13).

Cell isolation and T-cell enrichment. Gut LPMC were isolated as described below. Intestinal tissue was washed extensively with RPMI 1640 medium, and all visible Peyer's patches were removed with a scissor. The intestine was opened longitudinally, cut into 5-mm pieces, and then incubated in 0.5 mM EDTA in calcium-and-magnesium-free Hanks' solution for 20 min at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. This was repeated after thorough washing. Tissue then was incubated for 20 min at 37°C in 20 ml RPMI containing 10% fetal calf serum (FCS), 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all from GIBCO) and 1 mg/ml collagenase (co130; Sigma). At the end of the incubation, the tissue was subjected to further mechanical disruption using a 1-ml syringe. To remove debris, the LPMC preparations were washed through a prewet gauze layered in a funnel with RPMI. Then, the LPMC were washed once and were sieved through a prewet 2-cm nylon wool column gently packed into a 10-ml syringe. After washing, cells (up to 2×10^7) were layered onto a column of Percoll with a 30%:70% gradient. Cells were spun at $2,200 \times g$ at room temperature for 20 min. The LPMC collected from the 30:70 interface were washed and maintained on ice until used. Cell viability was 90% as determined by eosin Y exclusion.

LP T cells (Thy1.2⁺) were isolated by positive selection using antibody-coated, paramagnetic beads as described by the manufacturer (Dyna, Inc., New Hyde Park, NY). Flow cytometry was used after each separation to assure appropriate recovery and purity (>98%) of the Thy⁺ T cells. The Thy⁻ cells contained all the other expected leukocyte subsets and were thoroughly depleted of T cells (<1%).

Cell culture. For cytokine analysis, cells were cultured for 48 h in 96-well microtiter plates (Corning, Cambridge, MA) with 200 μ l of medium (5×10^5 cells/well) at 37°C. The culture medium was RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all from GIBCO). For most experiments, the cells were cultured alone or with anti-CD3 (2C11; ATCC) and anti-CD28 monoclonal antibody (MAB) (PharMingen, San Diego, CA) (each at 1 μ g/ml). Isolated T cells were cultured in wells previously coated overnight with anti-CD3 and -CD28 MAB. For IL-12 analysis, cells were cultured with CpG oligonucleotide (ODN 1826; TCCAT-GACGTCCTGACGTT) (Coley Pharmaceutical Group, Wellesley, MA) at 0.6 μ g/ml to stimulate production. Blocking IL-10, experiments used anti-IL-10R MAB at 2.5 μ g/ml (BD PharMingen).

Flow cytometric analysis. LPMC were washed twice and adjusted to 10^7 cells/ml in fluorescence-activated cell sorter (FACS) buffer (Hanks' balanced salt

solution containing 1% FCS and 0.02% sodium azide). The cell suspensions then were dispensed into microcentrifuge tubes, each containing 10^6 cells in 100 μ l FACS buffer, and stained with saturating amounts of conjugated antibodies for 30 min at 4°C. Following staining, cells were washed twice. Stained cells were analyzed on a Becton Dickinson FACS 440 flow cytometer (Mountain View, CA).

Before addition of labeled MAB, each tube received 1 μ g 2.4G2 antibody (anti-Fc γ R) (ATCC) to block nonspecific binding of conjugated antibodies to Fc receptors. The other MABs used for staining were anti-CD4-Cy5 (RM2511) (CalTag, Burlingame, CA), anti-CD8a-PE (53-6.7) (Sigma), anti-Thy1.2-fluorescein isothiocyanate (TS) (Sigma), and anti-CD25 (PC61) (PharMingen, San Diego, CA).

For IFN- γ and IL-4 intracellular cytokine staining, LPMC from control or *H. polygyrus*-infected mice were stimulated with anti-CD3 and anti-CD28 MABs for 14 h with brefeldin A (Golgi Plug; BD PharMingen) added at a 1-ml/ml end concentration during the last 12 h of cell culture. Culture cells were suspended (2×10^7 cells/ml in FACS buffer) and Fc receptors blocked with 2.4G2 MAB. The cells were stained with anti-Thy1.2 fluorescein isothiocyanate (BD PharMingen). To identify cytokine-secreting cells, cells were costained with anti-IL-4 phycoerythrin and anti-IFN- γ phycoerythrin-Cy7 (BD PharMingen) using the Cytofix/Cytoperm kit (BD PharMingen) according to the manufacturer's instructions. To detect IL-12 (p40)-positive cells, LPMC were stimulated with lipopolysaccharide or CpG oligonucleotide ODN 1826 and stained with anti-CD11c and anti-IL-12(p40) (BD PharMingen) as described above. Cells were analyzed on a FACS 440 flow cytometer (BD Biosciences, Mountain View, CA).

ELISAs. An enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of various cytokines in the supernatants. To measure IFN- γ , plates were coated with a MAB to IFN- γ (HB170; ATCC) and incubated with a supernatant. IFN- γ was detected with polyclonal rabbit anti-IFN- γ (gift from Mary Wilson, University of Iowa) followed by biotinylated goat anti-rabbit IgG (Accurate Chemical Co., Westbury, NY). Color development used streptavidin-horseradish peroxidase (Zymed, San Francisco, CA) and the tetramethylbenzidine substrate (Endogen, Woburn, MA), and plates were read at 490 nm. IL-4 was captured with 11B11 (HB191; DNAX Research Institute, Palo Alto, CA) and detected with biotinylated BVD6 (provided by Kevin Moore and John Abrams, DNAX). IL-5 was captured with TRFK5 and detected with biotinylated TRFK4 (Robert Coffman, DNAX). The IL-12 ELISA used anti-IL-12 MAB to capture p70 plus p40 (C15.6; Wistar Institute, Philadelphia, PA). Detection used biotinylated IL-12 MAB (C17-8; Endogen). IL-13 was measured using goat anti-murine IL-13 (AF-413-NA; R&D Systems, Minneapolis, MN) for capture and biotinylated monoclonal rat anti-mouse IL-13 (MAB413; R&D Systems) for detection. IL-10 was captured with anti-IL-10 MAB (MAB417; R&D Systems) and detected with biotinylated MAB (BAF417; R&D Systems).

MABs to IFN- γ , IL-4, IL-5, and capture IL-12 were from cell lines maintained in our laboratory. These MABs were purified from culture supernatants by ammonium sulfate precipitation.

Statistical analysis. Data are means $\pm$ standard errors (SE) of multiple determinations. The difference between two groups was analyzed using Student's *t* test. *P* values of <0.05 were considered significant.

RESULTS

***Heligmosomoides polygyrus* colonization protects from TNBS colitis.** TNBS colitis is characterized by ulcer formation, infiltration of the LP with chronic inflammatory cells, and transmural lymphocytic inflammation (22). The colitis results from an excessive Th1-type mucosal response. Blocking IL-12 or IFN- γ activity inhibits the inflammation (23), while blocking IL-10 worsens the disease (15). Previously we showed that mice systemically exposed to eggs of the trematode *Schistosoma mansoni* were protected from developing TNBS colitis (13). We tested if exposure to the nematode *H. polygyrus* protects mice from TNBS colitis.

Animals were inoculated with *H. polygyrus*. Two weeks later, animals were challenged with TNBS in alcohol given intrarectally. Control animals had severe colitis, while animals exposed to *H. polygyrus* had minimal intestinal inflammation (Fig. 1).

***Heligmosomoides polygyrus* does not alter histology in the distal intestine.** The intestinal helminth *H. polygyrus* lives ex-

FIG. 1. Mice colonized with *H. polygyrus* are protected from TNBS colitis. Mice were inoculated with *H. polygyrus*, while control mice received only sham inoculation. Two weeks later, they received TNBS in 50% ethanol given rectally to induce colitis. Colitis severity was assessed 4 days later. The inflammation was scored on a 0-to-4 scale. Previous experiments determined that the height of the inflammation occurred at day 4. Data are means $\pm$ SE from 10 animals studied in 2 separate experiments. Control versus *H. polygyrus*, $P < 0.01$.

clusively in the duodenum of mice. Mice were inoculated with 200 infective *H. polygyrus* larvae. *Heligmosomoides polygyrus* was visible in the duodenum at week 2 postinoculation. The terminal ileum and colon were examined histologically at week 1, week 2, and week 4 postinfection. In distal regions of the intestines, there were no morphological alternations or evidence of inflammation at any time point (data not shown).

Isolated, dispersed LPMC from the distal small intestine were examined for T-cell composition using flow cytometry. LPMC from mice carrying *H. polygyrus* for 2 weeks contained CD4⁺ Thy1.2⁺ T cells in proportions similar to that for LPMC from uninfected WT controls (Table 1). However, mice with *H. polygyrus* had fewer CD8⁺ Thy1.2⁺ T cells. Few T cells in either animal group expressed CD25.

***Heligmosomoides polygyrus* inhibits mucosal Th1 cytokine production.** Blocking IFN- γ or IL-12 protects mice from TNBS colitis. Next, we examined the effect of *H. polygyrus* on the capacity of the normal distal intestinal mucosa to produce these Th1 cytokines. LPMC from the terminal ileum of mice without *H. polygyrus* released IFN- γ and IL-12 (p40) (Fig. 2) when cultured in vitro. Anti-CD3/CD28 MAb stimulation strongly enhanced IFN- γ secretion. IL-12 (p40) was detected only after CpG oligonucleotide stimulation.

Age-matched mice were colonized with *H. polygyrus* for 2 weeks. After anti-CD3/CD28 stimulation, only small amounts of IFN- γ were detected in the culture supernatants of LPMC isolated from these mice (Fig. 2). None was constitutively released. IL-12 (p40) was not detected even after CpG stimulation.

Intestinal LP T cells from uninfected mice were isolated using paramagnetic beads. They produced large amounts of IFN- γ with anti-CD3/CD28 MAb stimulation (Fig. 3A). How-

ever, LPMC devoid of T cells made none. Intracytoplasmic staining for IFN- γ in unfractionated LPMC showed that only Thy1.2⁺ cells made this cytokine in response to anti-CD3/CD28 MAb stimulation (Fig. 3B), suggesting that LP T-cell IFN- γ production was regulated by colonization with *H. polygyrus*.

***Heligmosomoides polygyrus* promotes mucosal Th2 cytokine secretion.** IL-4, IL-5, and IL-13 are Th2 cytokines. We examined the effect of *H. polygyrus* on mucosal IL-4, IL-5, and IL-13 production in the distal intestinal mucosa. LPMC isolated from healthy WT mice secreted little or no IL-4, IL-5, or IL-13 constitutively. LPMC from mice bearing *H. polygyrus* produced similar amounts spontaneously. However, T-cell activation with anti-CD3/CD28 MAb substantially increased IL-4, IL-5, and IL-13 secretion from LPMC derived from the mice colonized with *H. polygyrus*, which was not the case with LPMC from control animals (Fig. 4).

LP T cells from mice bearing *H. polygyrus* were isolated using paramagnetic beads and cultured with or without anti-CD3/CD28 MAb stimulation to study IL-4 and IL-5 secretion. LPMC devoid of T cells were cultured also. The isolated LP T cells produced substantial amounts of IL-4 (Fig. 5A) and IL-5 (data not shown) only after anti-CD3/CD28 stimulation. LPMC depleted of T cells did not respond to anti-CD3/CD28 stimulation with enhanced IL-4. Intracytoplasmic staining of unfractionated LPMC for IL-4 showed that only Thy1.2⁺ cells made this cytokine in response to anti-CD3/CD28 MAb stimulation (Fig. 5B), suggesting that LP T cells acquired the capacity to make IL-4 after colonization with *H. polygyrus*.

LP T cells can make large amounts of IL-10 after *H. polygyrus* exposure. Also examined was IL-10 production. Unfractionated LPMC from mice with or without *H. polygyrus* spontaneously secreted IL-10 at similar rates. Anti-CD3/CD28 augmented IL-10 production in both groups, but LPMC from *H. polygyrus*-colonized animals made substantially more (>8-fold) (Fig. 6A).

Isolated LP T cells from either group did not spontaneously secrete IL-10. T cells stimulated with anti-CD3/CD28 MAb made IL-10. However, the LP T cells from mice colonized with *H. polygyrus* released 20-fold more IL-10 than those of the uninfected control mice (Fig. 6B). LPMC depleted of T cells made small amounts of IL-10, whose production levels were similar between the groups. IL-10 secretion from LPMC de-

TABLE 1. Relative proportions of CD4⁺ CD8⁺ or CD4⁺ CD25⁺ T cells in dispersed LPMC or mesenteric lymph nodes from WT mice

Cell type	Subset	% Cells in subset for mice with ^a :	
		No <i>H. polygyrus</i>	<i>H. polygyrus</i>
LPMC	Thy1.2 ⁺ CD4 ⁺	25.0 $\pm$ 5.8	22.3 $\pm$ 8.2
	Thy1.2 ⁺ CD8 ⁺	12.8 $\pm$ 2.9	3.5 $\pm$ 0.6 ^c
	CD4 ⁺ CD25 ⁺	3.2 $\pm$ 1.0	3.7 $\pm$ 0.6
MLN ^b	Thy1.2 ⁺ CD4 ⁺	26.0 $\pm$ 6.4	24.2 $\pm$ 1.3
	Thy1.2 ⁺ CD8 ⁺	14.9 $\pm$ 5.2	17.9 $\pm$ 2.8
	CD4 ⁺ CD25 ⁺	2.0 $\pm$ 0.9	2.9 $\pm$ 0.1

^a Data are mean determinations $\pm$ standard deviations from two or three separate experiments.

^b Mesenteric lymph nodes.

^c Results for no-*H. polygyrus* group versus *H. polygyrus*-infected group, $P < 0.05$.

FIG. 2. *H. polygyrus* inhibits mucosal IFN- γ and IL-12 (p40) responsiveness. Healthy C57BL/6 mice were inoculated with 200 *H. polygyrus* larvae by oral gavage, while control mice received only sham inoculation. Two weeks later, LPMC from the terminal ileum of either *H. polygyrus*-colonized or control animals (*H. poly* versus Control) were cultured (0.5×10^6 cells/well) for 48 h with or without anti-CD3/CD28 (α CD3) to stimulate IFN- γ production (A) or with CpG oligonucleotide (0.6 μ g/ml) to stimulate IL-12 (p40) secretion (B). Supernatants were assayed for IFN- γ and IL-12 (p40) content using ELISAs. Data are means $\pm$ SE of nine determinations from three independent experiments. Control versus *H. polygyrus*: anti-CD3-stimulated IFN- γ , $P < 0.01$; CpG-stimulated IL-12 p40, $P < 0.01$.

pleted of T cells did not increase with anti-CD3/CD28 stimulation (data not shown).

IL-10 helps limit mucosal IFN- γ and IL-12 production. IL-10 is an important regulatory cytokine that can help limit Th1 cytokine production. Experiments determined if the heightened IL-10 secretion helped limit mucosal Th1 cytokine production in WT mice bearing *H. polygyrus*. LPMC isolated from WT mice with or without *H. polygyrus* were cultured for 48 h in the presence or absence of anti-IL-10R MAb to block

endogenous IL-10 activity. Some cultures contained anti-CD3/CD28 MAbs to stimulate T-cell secretion, while others had CpG oligonucleotide to enhance IL-12-related cytokine production. Culture supernatants then were examined for IFN- γ and IL-12 (p40) secretion. As expected, LPMC from worm-infested mice cultured without anti-IL-10R made little IFN- γ and IL-12 p40 compared to uninfected controls (Fig. 7). However, IL-10R blockade restored IFN- γ and IL-12 p40 production to normal levels (Fig. 7).

FIG. 3. T cells are the source of anti-CD3/CD28-stimulated IFN- γ in LPMC from uninfected mice. (A) LP T cells (T cells) or LPMC depleted of T cells (Non T) derived from uninfected WT mice were cultured (5×10^5 cells/well) for 48 h with or without adherent anti-CD3/CD28 MAbs (α CD3). Culture supernatants were assayed for IFN- γ after the incubation. Data are means $\pm$ SE of nine determinations from three independent experiments. (B) Unfractionated LPMC from uninfected WT mice were cultured as described in the legend for Fig. 2 and then assayed for expression of IFN- γ by intracellular flow cytometry using a lymphoid gate. Results are representative of two independent experiments.

FIG. 4. *Heligmosomoides polygyrus* promotes mucosal IL-4, IL-5, and IL-13 production. C57BL/6 mice were inoculated with 200 *H. polygyrus* larvae (*H. poly*) and LPMC cultured as described in the legend for Fig. 2. Control mice did not receive *H. polygyrus*. Next, supernatants were assayed for IL-4, IL-5, and IL-13 content using ELISAs. Data are means $\pm$ SE of nine determinations from three independent experiments. Control versus *H. polygyrus*: IL-4, $P < 0.05$; IL-5, $P < 0.01$; IL-13, $P < 0.01$.

IL-10R blockade partially inhibits the protection afforded by helminth colonization against TNBS colitis. Blockade of IL-10R in vitro restores IFN- γ and IL-12 p40 production by LP cells from colonized mice. Treatment with monoclonal anti-IL-10 antibody inhibits the protection against TNBS colitis afforded by feeding haptenated colonic proteins (15). We determined if IL-10R blockade would abrogate helminth-associated protection against TNBS colitis (Fig. 8). Treatment with blocking monoclonal anti-IL-10R antibody at 0.5 mg/mouse slightly worsened TNBS colitis compared to results for mice treated with isotype control antibody, but this did not achieve

statistical significance ($P = 0.41$). Colonization with helminths provides good protection ($P < 0.001$) in the presence of isotype control antibody compared to results for worm-free control antibody-treated mice. Treatment with blocking anti-IL-10R antibody significantly worsened colitis in helminth-colonized mice but did not completely abolish protection.

LP CD4⁺ T cells from *H. polygyrus*-colonized mice inhibit splenic T-cell IFN- γ production. The ability to restore LP IFN- γ production with IL-10R blockade suggests that there is active regulation of IFN- γ production by LP cells of *H. polygyrus*-colonized mice. We examined the effect on IFN- γ produc-

FIG. 5. LP T cells are the source of anti-CD3/CD28 (α CD3)-stimulated IL-4 in colonized mice. C57BL/6 mice were inoculated with 200 *H. polygyrus* larvae by oral gavage. (A) Two weeks later, LP T cells (T Cells) or LPMC depleted of T cells (Non-T) (5×10^5 cells/well) were cultured as described in the legend for Fig. 2. Data are means $\pm$ SE of nine determinations from three independent experiments. (B) Unfractionated LPMC from *H. polygyrus*-colonized WT mice were cultured as described in the legend for Fig. 3 and then assayed for expression of IL-4 by intracellular flow cytometry using a lymphoid gate as described in the legend for Fig. 3. Results are representative of two independent experiments.

FIG. 6. *Heligmosomoides polygyrus* induces mucosal IL-10 production. Mice were inoculated with *H. polygyrus*, while control mice received only sham inoculation. Two weeks later, unfractionated LPMC (Cells) from either *H. polygyrus*-colonized or control animals (H. poly versus Control) were cultured as described in the legend for Fig. 2 with or without anti-CD3/CD28 MAbs (α CD3). Supernatants then were assayed for IL-10 by ELISA. In other experiments, LP T cells or LPMC depleted of T cells from mice bearing *H. polygyrus* were cultured as described in the legend for Fig. 2 to study IL-10 secretion (B). Data are means $\pm$ SE for six determinations from two independent experiments. Control versus *H. polygyrus* anti-CD3-stimulated IL-10, $P < 0.01$ for either unfractionated or T-cell groups.

tion of mixing LP T cells from worm-colonized mice with isolated splenic T cells from worm-free mice. Normal splenic T cells mixed with irradiated antigen-presenting cells (APC) and stimulated with anti-CD3 made IFN- γ (Fig. 9). Addition of LP T cells to these cultures completely inhibited IFN- γ production. Inhibition also occurred when the LP T cells were separated from the splenic T cells by a semipermeable membrane, demonstrating that cell contact was not required for this regulation. IL-10R blockade with anti-IL-10R MAb did not enhance IFN- γ production by anti-CD3-stimulated splenic T cells mixed with APC or LP T cells cultured with soluble anti-CD3 but separated from APC by a semipermeable membrane. IL-10R blockade of cocultured LP T and splenic T cells partially restored IFN- γ production. LP cells from *H. polygyrus*-colo-

nized mice comprise 22.3% CD4 $^{+}$ cells and 3.5% CD8 $^{+}$ T cells (Table 1). LP CD4 $^{+}$ but not CD8 $^{+}$ T cells inhibited splenic T-cell IFN- γ production (Fig. 10).

DISCUSSION

IBD results from dysregulated intestinal mucosal immune responses that injure the host. Epidemiological evidence suggests that the environment present in highly developed countries increases the risk for IBD, whereas the environment in less-developed countries lowers the risk. One hypothesis to explain the epidemiological observations is that helminth exposure, common in less-developed countries, protects from IBD (11).

FIG. 7. Anti-IL-10R MAb (α IL10R) reverses both IFN- γ and IL-12 blockade. Mice were inoculated with *H. polygyrus*. Two weeks later, unfractionated LPMC from either control mice (Control) or *H. polygyrus*-colonized animals (H. poly) were cultured (0.5×10^6 cells/well) for 48 h with anti-CD3/CD28 MAbs to stimulate IFN- γ production (A) or CpG oligonucleotides (0.6 μ g/ml) to promote IL-12 p40 secretion (B). Some wells also contained blocking anti-IL-10R MAb. After the incubation, supernatants were assayed for IFN- γ and IL-12 p40. Data are means $\pm$ SE of eight determinations from four independent experiments. Control IFN- γ , $P < 0.05$; *H. polygyrus* IFN- γ , $P < 0.01$; control IL-12, $P < 0.05$; *H. polygyrus* IL-12, $P < 0.01$.

FIG. 8. Treatment with monoclonal anti-IL-10R antibody worsens TNBS colitis in *H. polygyrus*-colonized mice. Mice were inoculated with *H. polygyrus*, while control mice received only sham inoculation. Two weeks later, they received TNBS as described in the legend for Fig. 1 and were given either rat IgG (0.5 mg) (IgG Rx) or monoclonal rat anti-mouse IL-10R blocking antibody (0.5 mg) (α IL10R) by intraperitoneal injection. Colitis severity was assessed 4 days later as described in the legend for Fig. 1. Shown are representative photomicrographs from helminth-naïve mice treated with isotype control antibody (A), helminth-naïve mice treated with monoclonal anti-IL-10R antibody (B), helminth-colonized mice treated with isotype control antibody (C), or helminth-colonized mice treated with monoclonal anti-IL-10R antibody (D). Data are means $\pm$ SE from three separate experiments, each with four to five mice per group.

Mice treated with rectal instillation of the hapten TNBS develop severe colitis that shares features with CD (22). In this model, infiltrating LPMC and CD4⁺ T cells secrete high levels of TNF- α and IFN- γ . Treatment with anti-IL-12 MAb (23), anti-IFN- γ MAb, anti-TNF- α MAb, or recombinant IL-10 prevents the colitis (22), showing that the inflammation results from an overly vigorous Th1-type response induced by TNBS.

Helminths can protect mice from TNBS-induced colitis. Intraperitoneal exposure to nonviable schistosome ova protects

mice from TNBS colitis. This protective process requires IL-4 and is associated with a rise in IL-10 mRNA content in the colon (13). *Schistosoma mansoni* is a platyhelminth trematode that resides in the mesenteric veins draining the intestine. Similar protection from TNBS-type colitis also is seen with the nematode *Trichinella spiralis* (18) and the cestode *Hymenolepis diminuta* (16).

FIG. 9. LP T cells from colonized mice inhibit splenic T-cell IFN- γ production. Transwell experiments were performed with 96-well Transwell plates. Splenic T cells (Spl T) (3×10^4 cells/well) were mixed with equal numbers of irradiated APC and placed in the outer chamber. LP T cells (6×10^4 cells/well) from *H. polygyrus*-infected mice were either added directly to the outer chamber or placed in the inner chamber of the Transwell system. Some chambers also received blocking anti-IL-10R (α IL10R) MAb (1 mg/ml). The ratio of splenic T cells to the LP T cells was 1:2. Cells were cultured for 48 h. IFN- γ was measured in culture supernatants by ELISA. Data are means $\pm$ SE of eight determinations from two independent experiments. *, $P < 0.05$.

FIG. 10. LP CD4⁺ but not CD8⁺ T cells inhibit splenic T-cell IFN- γ production. LP T cells from *H. polygyrus*-infected mice were enriched for the CD8⁺ or CD4⁺ subset as described in Materials and Methods. These cells were mixed with the splenic responder T cells (Spl T) at a 2:1 ratio. Cells were cultured for 48 h, and then IFN- γ was measured in culture supernatants by ELISA. Data are means $\pm$ SE of 12 determinations from 3 independent experiments. Spl T versus Spl T + CD4⁺ LP, $P < 0.01$; Spl T versus Spl T + CD8⁺ LP, $P > 0.05$.

This study used *H. polygyrus* (14), which is a nematode helminth ideally suited for this investigation. *Heligmosomoides polygyrus* is a natural intestinal helminth of mice that inhabits only the first part of the small intestine (duodenum). Mice are colonized with this organism by the placing of larvae in the stomach through gastric lavage. The larvae migrate to the duodenum, enter the submucosa, mature, and then emerge as adult worms that persist in the intestinal lumen. They remain in the duodenum and do not invade through the intestinal wall. Having an agent that colonized only the duodenum without inducing inflammation or anatomical changes in the ileum or colon was an important advantage. During *H. polygyrus* colonization, cytometric analysis of isolated LPMC from the ileum or colon revealed only minimal alterations in cell composition.

However, our data showed that helminths living in the duodenum severely restrict the capacity of intestinal mucosa to mount a Th1-type cytokine response (IFN- γ and IL-12 p40-related cytokines) in more-distal regions of the bowel. Mucosal IFN- γ production was T cell dependent, suggesting that the helminth exposure inhibited LP Th1-cell function. Intracytoplasmic cytokine staining of LP T cells showed that colonization with *H. polygyrus* did not alter the frequency of CD4⁺ IFN- γ ⁺ T cells in the population (data not shown). This suggested that the inhibition of LP Th1 function was an active process rather than simple exclusion of Th1 cells from the mucosal cell population.

IL-10 is a mediator with strong immune modulatory functions (21). IL-10 inhibits macrophage and dendritic cell function and suppresses production of various important proinflammatory cytokines, such as TNF- α , IL-12, IL-1, nitric oxide, and various chemokines. This subsequently affects T-cell function. It also inhibits the CD28 costimulatory pathway, promoting T-cell tolerance (2). Mice with disruption of the IL-10 gene spontaneously develop severe Th1-type colitis (26). Reconstitution of Rag1-deficient mice (which lack T or B cells) with T cells from IL-10^{-/-} animals results in colitis, suggesting that T-cell IL-10 is required for intestinal mucosal immune homeostasis (7).

Thus, we studied the possible role of IL-10 in worm-induced, local restriction of mucosal "Th1"-cell function. It was found that blocking IL-10R restored IFN- γ and IL-12 production in LPMC cultured in vitro. This suggested that IL-10 has an important role in this local regulatory process. Also, restoration of Th1 cytokine production following IL-10 blockade showed that cytokine regulation rather than Th1 cell deletion was the mechanism of action. Treatment of mice with blocking monoclonal anti-IL-10R antibody significantly worsened TNBS colitis in *H. polygyrus*-colonized mice. This shows the importance of the IL-10 regulatory circuit, which may also serve to inhibit excessive Th2 responses in the mucosa (1, 5). Although blocking IL-10 signaling increased LPMC IFN- γ and IL-12 p40 production in vitro, it did not worsen TNBS colitis in helminth-naïve mice. This suggests that other immunoregulatory circuits in addition to IL-10 serve to limit colitis. Anti-IL-10R treatment of helminth-colonized mice did not permit colitis to return to the level induced in worm-naïve mice. This may be due to incomplete IL-10R blockade or more likely activity by other regulatory mechanisms augmented by helminth exposure. One such regulatory mechanism could involve TGF- β . Increased TGF- β signaling suppresses TNBS colitis (8, 15), and LPMC

TGF- β production is augmented by helminth exposure (17). *H. polygyrus* can also induce regulatory CD8⁺ T cells in the intestinal mucosa that can limit colitis (20). Thus, helminths stimulate several immunoregulatory pathways, all of which may limit disease.

While *H. polygyrus* exposure limited the capacity of LP T cells to produce IFN- γ , the production of IL-4, IL-5, and IL-13 was enhanced. Protective immunity to these parasites is CD4⁺ T cell dependent (14). The normal C57BL/6 host eliminates these worms by mounting a Th2 response (e.g., IL-4 and IL-13). IL-4 induces murine B-cell differentiation toward the Th2 phenotype (IgE and IgG1) and helps prevent Th1-cell differentiation. Although not studied, it remains possible that IL-4 and IL-13, the two critically important Th2 cytokines, also are important in control of the mucosal Th1 response.

Cytokines come from various sources. It is necessary to appreciate the origin of regulatory cytokines that govern Th1 cytokine production to more fully understand the regulatory circuitry active at mucosal surfaces. For instance, IL-10 can derive from T cells, B cells, or APC (21), while IL-4 frequently comes from Th2 cells, mast cells, or eosinophils (4, 31). It was found that worm exposure induced mucosal T cells to become a major source for IL-10 and IL-4.

The greatly increased capacity of T cells to make IL-10 in the mucosa following worm exposure suggests that helminths induce some type of regulatory T cell locally within the intestine that may limit Th1 responsiveness. The failure to detect appreciable increases in CD25⁺ T cells may imply an absence of the "natural" regulators in the mucosa or the fact that these cells represent a subset of LP CD25⁺ T cells that expands at the expense of nonregulatory CD25⁺ T cells. We found that LP CD4⁺ T cells isolated from *H. polygyrus*-colonized mice could block splenic T cells from control mice from making IFN- γ . This inhibition did not require cell contact, suggesting that it was not dependent upon classical CD4⁺ CD25⁺ natural regulatory T-cell activity. In splenic T cells cocultured with LP T cells, blockade of IL-10 signaling restored splenic T-cell IFN- γ production. This suggests that helminth colonization induces an LP T-cell population with Tr1 activity.

Recently we showed that colonization with *H. polygyrus* can reverse established colitis in IL-10-deficient mice (10). In that model system, *H. polygyrus* inhibited LPMC IFN- γ and IL-12 production in the absence of IL-10. The current study explores mucosal T-cell-regulatory activity in mice with intact immunity. In contrast to the case with IL-10-deficient mice, inhibition of IFN- γ production by LPMC from WT *H. polygyrus*-colonized mice is IL-10 dependent. This suggests that helminth exposure induces multiple regulatory circuits that may vary depending on the host. Indeed, we have found that helminths induce T-cell immunoregulatory TGF- β (17), PGE2 (data not shown), and a CD8⁺ T cell that inhibits T-cell proliferation (20).

In summary, we show that a live nematode parasite with a strictly enteric life cycle can protect mice from TNBS colitis. A previous study showed that *H. polygyrus* induces Th2 cytokine mRNA expression in the mesenteric lymph nodes and Peyer's patches of the host (30). We now demonstrate that *H. polygyrus* living exclusively in the duodenum inhibits Th1 responses in distant regions of the intestine. This inhibition coincided with induction of LP T cells that make Th2 and the regulatory cytokine IL-10. IL-10 actively suppressed proinflammatory cy-

tokine production (IFN- γ and IL-12 p40) by LPMC and was responsible for some of the protection from TNBS colitis afforded by helminthic exposure. Worms appear to induce regulatory-type T cells in the LP of distal bowel regions.

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